AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

MICROBIAL IMMUNITY AND VACCINES

Characterization of *Plasmodium berghei* Pbg37 as Both a Pre- and Postfertilization Antigen with Transmission-Blocking Potential

Fei Liu,[a] Li Li,[a] Wenqi Zheng,[b] Yiwen He,[a] Yaru Wang,[a] Xiaotong Zhu,[a] Takafumi Tsuboi,[c] Liwang Cui,[a,d] Meilian Wang,[a] Yaming Cao[a]

[a]Department of Immunology, College of Basic Medical Sciences, China Medical University, Shenyang, Liaoning, China

[b]Laboratory of Surgery, The Affiliated Hospital, Inner Mongolia Medical University, Hohhot, China

[c]Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Ehime, Japan

[d]Department of Entomology, Pennsylvania State University, University Park, Pennsylvania, USA

ABSTRACT Transmission-blocking vaccines (TBVs) interrupting malaria transmission are an integrated tool for malaria eradication. We characterized a sexual-stage-specific gene (PBANKA_060330) from *Plasmodium berghei* and studied its potential for use as a TBV. This gene, referred to as *pbg37*, encodes a protein of 37 kDa with a signal peptide and multiple transmembrane domains and is preferentially expressed in gametocytes. A recombinant Pbg37 (rPbg37) protein targeting the N-terminal 63 amino acids (amino acids 26 to 88) expressed in bacteria elicited strong antibody responses in mice. Western blotting demonstrated Pbg37 expression in gametocytes, zygotes, and, to a lesser extent, ookinetes and its predominant association with the membranes of gametocytes. Indirect immunofluorescence assay showed an abundant surface localization of Pbg37 on gametes and zygotes but reduced amounts on retorts and ookinetes. Knockout of *pbg37* (Δ*pbg37*) led to a considerable reduction in gametocytemia, which translated into a ~92.1% decrease in the oocyst number in mosquitoes. Deletion of *pbg37* had a more substantial influence on the development and maturation of microgametocytes. As a result, the Δ*pbg37* lines exhibited a higher female/male gametocyte ratio, fewer mature male gametocytes, and defects in the exflagellation of mature microgametocytes. To test the transmission-blocking potential of Pbg37, an *in vitro* ookinete assay showed that the major inhibitory effects of anti-Pbg37 antiserum were on the exflagellation and fertilization processes. Direct feeding of mosquitoes on mice immunized with rPbg37 or a control protein showed that rPbg37-immunized and *P. berghei*-infected mice had a significant reduction (49.1%) in oocyst density compared to the controls. The conservation of this gene in *Plasmodium* warrants further investigations in human malaria parasites.

KEYWORDS *Plasmodium berghei*, gametocyte, gamete, ookinete, transmission-blocking vaccine, prefertilization, postfertilization

Malaria is a vector-borne disease caused by protozoan parasites in the genus *Plasmodium* and is transmitted through female *Anopheles* mosquitoes. It remains one of the devastating infectious diseases and carries a serious social and economic burden in developing countries (1). In the past decade, significant progress in malaria control has led to renewed interest in malaria elimination (2). Yet, for the realization of this goal, countries where malaria is endemic face a number of technical challenges. Given that the malaria life cycle involves both humans and mosquitoes, integrated control measures targeting stages in both hosts are desired. Current malaria management relies heavily on effective chemotherapy to treat infected patients and on vector

Received 31 October 2017 **Returned for modification** 5 February 2018 **Accepted** 26 May 2018

Accepted manuscript posted online 4 June 2018

Citation Liu F, Li L, Zheng W, He Y, Wang Y, Zhu X, Tsuboi T, Cui L, Wang M, Cao Y. 2018. Characterization of *Plasmodium berghei* Pbg37 as both a pre- and postfertilization antigen with transmission-blocking potential. Infect Immun 86:e00785-17. https://doi.org/10.1128/IAI.00785-17.

Editor John H. Adams, University of South Florida

Address correspondence to Meilian Wang, Wangmeilian_m@hotmail.com, or Yaming Cao, ymcao@mail.cmu.edu.cn.

control, such as through the use of insecticide-treated bed nets and indoor residual sprays. However, these measures are compromised by the emergence of drug-resistant malaria parasites and insecticide-resistant mosquitoes (3, 4). Whereas vaccine is considered a cost-effective tool for eliminating malaria, the leading vaccine, RTS,S/AS01, a subunit vaccine targeting the circumsporozoite protein, has been proven to be only partially effective (5). Current vaccine development efforts explore antigens of multiple stages of the malaria parasite life cycle and have included the development of a transmission-blocking (TB) vaccine (TBV) that targets sexual development (6). TBVs aim to induce immune responses that interfere with parasite transmission to the mosquito and, consequently, block disease spread between humans (7). Despite their importance, only two candidates, Pfs25 and Pfs230, have reached the preclinical phase in the Malaria Vaccine Initiative's development pipeline (www.malariavaccine.org).

TBV candidates can be antigens expressed on gametocytes and gametes (prefertilization) or on zygotes and ookinetes (postfertilization) or even antigens expressed by the mosquito midgut (7–9). The well-documented presence of naturally acquired TB immunity in human populations living in areas where malaria is endemic probably resulted from the antigens released from dead gametocytes (7, 10), and thus, TBVs targeting the prefertilization antigens have the advantage of boosting natural immunity. Among the leading TBV candidates, P25/28, P48/45, P230, and HAP2 (11, 12), P25/28 is expressed from the female gamete until early oocyst development (13). Antiserum against Pfs25 has the ability to completely inhibit oocyst development in mosquitoes (13–15). Pfs230 and Pfs48/45 are localized on the outer membrane of gametes and play roles in gamete-gamete recognition and fertilization (16, 17). Pfs230 and Pfs48/45 antigen-specific IgGs show the ability to prevent oocyst formation, probably through lysis of gametes in a complement-dependent manner (18). HAP2, also referred to as GCS1 (generative cell specific 1) (19), is expressed on male gametocytes and microgametes and is indispensable for gamete fusion (20). Whereas anti-*Plasmodium berghei* HAP2 serum shows no influence on male gamete formation, it significantly interferes with ookinete and oocyst development (21). The mosquito midgut ligands carboxypeptidase B and alanyl aminopeptidase N1 (APN1) are TBV antigens expressed by the mosquito midgut (9, 22, 23). Antibodies against *Anopheles gambiae* APN1 possess strong TB activity against *P. falciparum* in different mosquito species (24). Although TBVs based on Pfs25 and Pvs25 have been evaluated in phase I clinical trials (25–27), TBV development still encounters a number of difficulties. For the lead TBV candidates, it often requires the expression of native proteins in the correct conformation in order to induce effective TB activity (25). Even so, most TBV candidates could not induce complete TB activity, and a successful TBV will probably need to combine antigens expressed in both the pre- and postfertilization stages in order to achieve complete blocking of transmission. With the limited number of TBV candidate antigens currently available, further efforts in TBV antigen discovery are required.

With our efforts in TBV antigen discovery and functional studies of genes during sexual development of the malaria parasites, we identified a sexual-stage gene that is highly conserved in *Plasmodium*. Characterization of this gene, designated *pbg37*, in the rodent parasite *P. berghei* revealed its expression in both the pre- and postfertilization stages. Deletion of this gene affected male gametocytogenesis and functions. Furthermore, immunization with the recombinant Pbg37 protein (rPbg37) resulted in a modest level of TB activity in direct mosquito feeding experiments.

RESULTS

Pbg37 is a highly conserved protein expressed in *Plasmodium* sexual stages. Using a bioinformatic scheme defined earlier (28), we searched PlasmoDB for (i) conserved proteins present in *Plasmodium* spp., (ii) the presence of a putative signal peptide, (iii) the presence of one or more predicted transmembrane regions, and (iv) proteins with abundant expression in gametocyte stages. This led to the identification of 13 genes with these characteristics and a transcript abundance in the top 97%

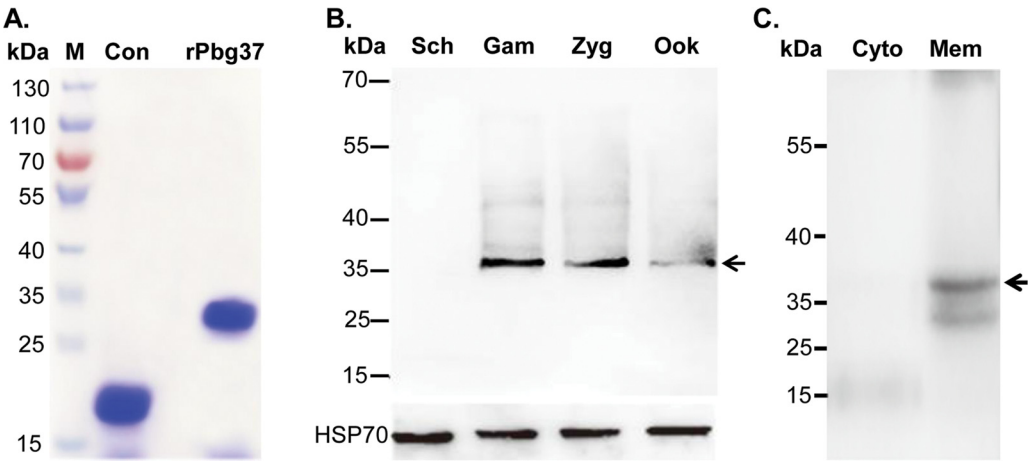

FIG 1 Expression of rPbg37 in *E. coli* and detection of Pbg37 expression during *P. berghei* development. (A) Purified rPbg37 fusion protein and the control Trx-His tag protein (Con) were subjected to electrophoresis on a 10% SDS-PAGE gel, and the proteins were stained with Coomassie brilliant blue. Lane M, molecular markers, with the sizes (in kilodaltons) indicated on the left. (B) Western blot analysis of lysates (10 μg/lane) of purified schizonts (Sch), gametocytes (Gam), zygotes (Zyg), and ookinetes (Ook) of the *P. berghei* parasites. Proteins were separated by 10% SDS-PAGE and probed with mouse polyclonal antisera against rPbg37 (arrow, top) and against *P. berghei* HSP70 as a protein loading control (bottom). (C) Western blot of the cytoplasmic (Cyto) and membrane (Mem) protein fractions of purified gametocytes probed with anti-Pbg37 serum, showing the 37-kDa protein band (arrow) present mainly in the membrane fraction.

percentile of the transcriptome sequencing (RNA-seq) data in the *P. berghei* gametocyte transcriptome (29). Among them, we selected PBANKA_060330 in *P. berghei* for detailed functional studies. This gene encodes 349 amino acids with a predicted molecular weight of 40 kDa. The predicted protein contains a putative signal peptide (amino acids 1 to 23), seven transmembrane regions, and one low-complexity region (see Fig. S1A in the supplemental material). Whereas BLAST analysis did not identify sequences homologous to this gene in organisms outside *Plasmodium*, this gene is highly conserved among *Plasmodium* species, as shown from the results of multiple-sequence alignments (Fig. S1B). Based on the predominant expression of this gene in the gametocyte stage of *P. berghei* from RNA-seq analysis and the predicted mature protein size of ~37 kDa after the cleavage of the signal peptide, we named this gene *pbg37*.

Recombinant Pbg37 induces high IgG titers in immunized mice. Because Pbg37 is predicted to have a signal peptide and multiple transmembrane domains, we selected a small, 63-amino-acid fragment between the signal peptide and the first transmembrane domain (amino acids 26 to 88) for protein expression in bacteria. Since *pbg37* is predicted to contain 12 introns, reverse transcription-PCR (RT-PCR) was performed using *P. berghei* gametocyte mRNA, and sequencing of the PCR product confirmed the annotated gene model. A His-tagged rPbg37-thioredoxin (Trx) fusion protein and the control Trx-His tag protein were purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography almost to homogeneity, as shown in an SDS-PAGE gel (Fig. 1A). The rPbg37-Trx fusion protein had a molecular weight of approximately 27 kDa, which agrees with its predicted size (~7 kDa of the Pbg37 fragment fused with the Trx and His tag in the vector).

Mice were immunized with the rPbg37 fusion protein or the control protein to produce polyclonal antibodies. To detect specific antibodies against the rPbg37 protein, the rPbg37 polypeptide in the fusion protein on the Ni-NTA column was released after digestion with enterokinase and used to coat enzyme-linked immunosorbent assay (ELISA) plates (Fig. S2A). The results showed that the second booster immunization induced a significantly higher titer in the rPbg37 immunization group than in the control group ($P < 0.0001$; Fig. S2B), and the antibody titer reached an endpoint dilution of 1:64,000 at 14 days after the final immunization (Fig. S2C). This result showed that under the immunization scheme used, the rPbg37 protein was highly immunogenic and induced specific antibodies in mice.

Pbg37 is expressed in sexual stages. Given the conservation of Trx in different organisms, we first performed Western blotting with antiserum against the control protein (Trx) to exclude the possibility of cross-reaction of the fusion Trx tag with parasite proteins. Equal amounts of lysates from purified schizonts, gametocytes, zygotes, and ookinetes were separated by SDS-PAGE. Immunoblotting with the control antiserum did not detect any specific parasite protein bands in lysates of the schizont, gametocyte, zygote, and ookinete stages (Fig. S3A). This is consistent with the results of a BLAST analysis, which showed that the two *P. berghei* Trx genes (PBANKA_1320900 and PBANKA_1365200) share only 35 and 33% homology, respectively, with Trx in the expression vector. In contrast, an ~37-kDa band was recognized by the anti-rPbg37 serum in the lysates of gametocytes, zygotes, and ookinetes, whereas no specific protein bands were detected in the lysate of schizonts, which agrees with the predominant expression of the *pbg37* mRNA in sexual stages (Fig. 1B). The size of the detected protein band is compatible with the predicted molecular weight of Pbg37 after cleavage of the putative signal peptide. Compared with the similar amounts of HSP70 protein detected in all parasite stages tested, the Pbg37 band in ookinetes appeared to be slightly lighter, suggesting that less Pbg37 was present in ookinetes.

Given that the predicted protein contains a signal peptide and transmembrane domains, we wanted to determine the localizations of the Pbg37 protein during sexual development. We first investigated whether Pbg37 was associated with the gametocyte plasma membrane by Western blotting. Separation of gametocyte proteins into cytosolic and membrane fractions and immunoblotting with anti-rPbg37 antiserum detected the ~37-kDa band mainly in the parasite plasma membrane fraction (Fig. 1C). Pbg37 localization was further studied by indirect immunofluorescence assay (IFA) using unfixed and fixed parasites with and without membrane permeabilization. Consistent with the Western blotting result, IFA of fixed and permeabilized cells detected Pbg37 expression in gametocytes, gametes, zygotes, and ookinetes but not in schizonts. Interestingly, a sex difference in Pbg37 expression was noted, as Pbg37 appeared to be more abundant in male than female gametocytes. In the exflagellating male gamete, Pbg37 was mostly associated with the residual body, while speckled fluorescence was also observed on the microgamete. In comparison, weaker fluorescence was observed on the macrogamete membrane, and the fluorescence had a spotty appearance. Whereas strong surface staining of the zygote was observed, the signal was gradually reduced during subsequent transformation to the retort form, and only residual fluorescence was observed on the ookinete stage. In mature gametocytes, the fluorescence was visible only in fixed and permeabilized cells and not in fixed nonpermeabilized cells or unfixed cells, indicating that Pbg37 is associated with the plasma membrane of gametocytes (Fig. 2 and S4A). In gametes, zygotes, retorts, and ookinetes, the fluorescence patterns were similar among unfixed cells as well as fixed cells with and without membrane permeabilization, indicating surface localization of at least the N-terminal domain of Pbg37 in these stages. Control serum showed undetectable levels of staining in these developmental stages (Fig. S3B).

***pbg37* is important for sexual-stage development, especially male gametocytes.** To determine the function of Pbg37 during *P. berghei* development, two *pbg37* knockout lines (KO1 and KO2) were generated using a double-crossover homologous recombination strategy from two independent transfection experiments (Fig. 3A). After transfection, parasites were selected with pyrimethamine, and *pbg37* deletion (Δ*pbg37*) was verified by integration-specific PCR (Fig. 3B). Phenotypic analysis of two Δ*pbg37* parasite clones (KO1 and KO2) was performed in parallel with the wild-type (WT) parasite strain. Consistent with the lack of *pbg37* expression in asexual erythrocytic stages, the Δ*pbg37* lines and the WT control showed no significant difference in daily asexual parasitemia (Fig. S5). However, the Δ*pbg37* lines showed significantly lower daily gametocyte densities than the WT parasite ($P < 0.0001$), though the dynamics of gametocytogenesis were similar. On day 3 postinfection (p.i.), when gametocytemia reached peak levels, there was an ~60% reduction in gametocytemia in the Δ*pbg37*

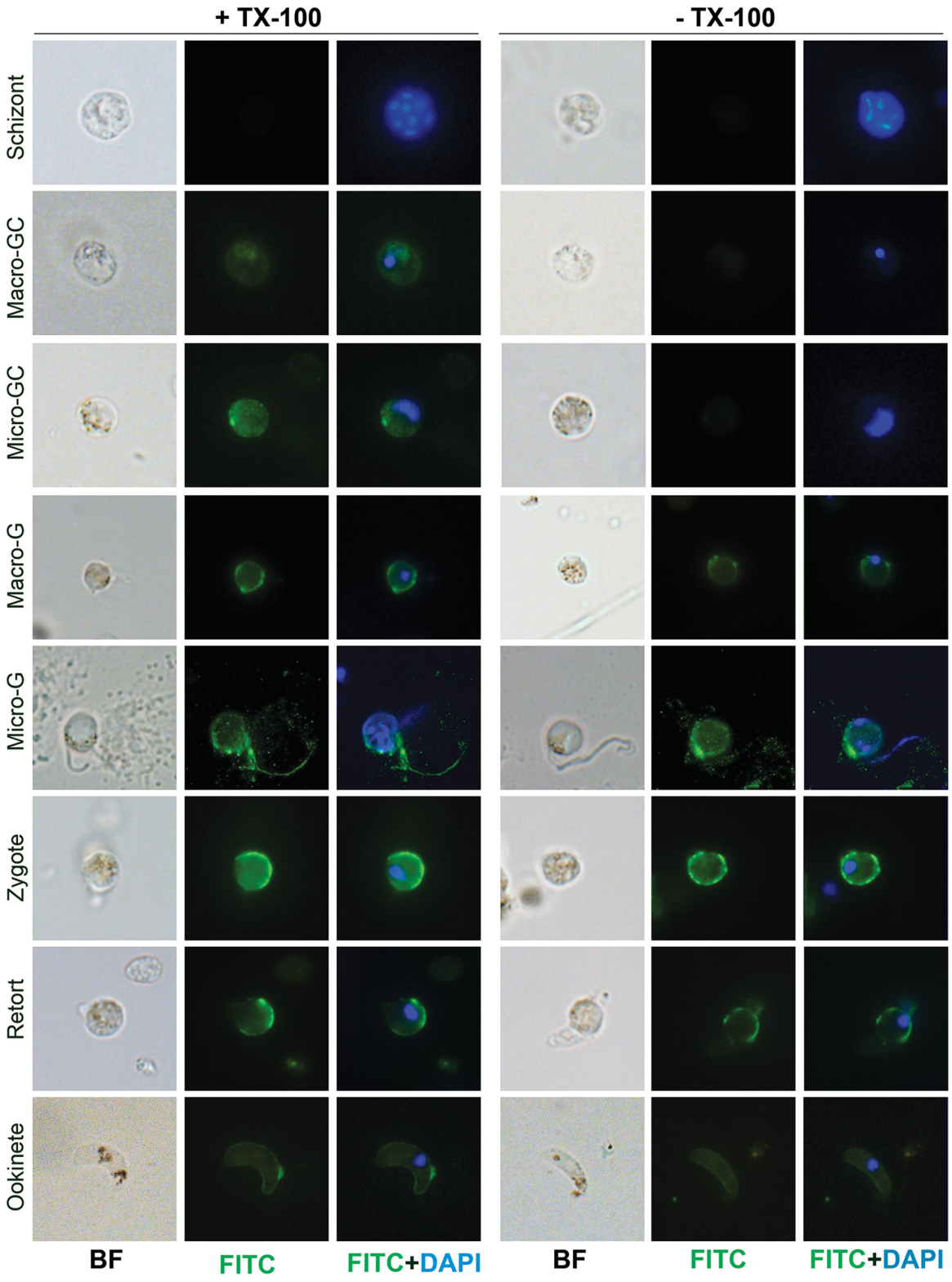

FIG 2 Localization of Pbg37 during parasite development in fixed *P. berghei* parasite cells. Representative IFA images of cells permeabilized with 0.1% Triton X-100 (+TX-100) and cells not permeabilized with Triton X-100 (−TX-100) show the localization of Pbg37 in the plasma membrane of different developmental stages of the parasite. Primary antibodies were polyclonal anti-Pbg37, whereas secondary antibody was FITC-conjugated anti-mouse IgG antibody. Nuclei were stained with DAPI. The images were obtained under the same conditions at a magnification of ×1,000. BF, bright field; FITC+DAPI, merge of the two images; Micro-G, microgamete; Macro-G, macrogamete; Micro-GC, microgametocyte; Macro-GC, macrogametocyte.

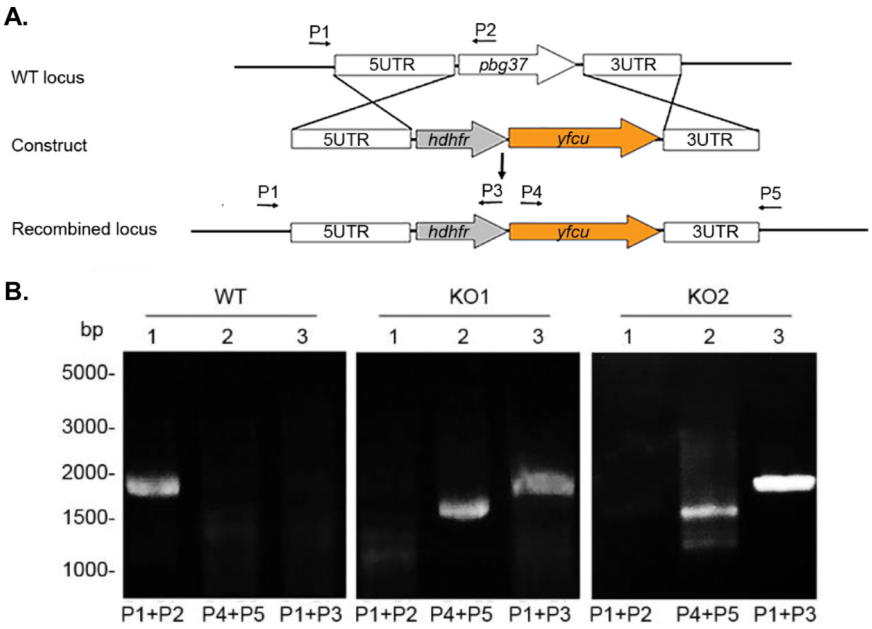

FIG 3 Generation of the Δ*pbg37* line. (A) Schematic representation of the WT *pbg37* locus, the transfection construct, and the recombined locus (not to scale). Primers P1 to P5 were used to detect either the WT locus or the replaced locus and are marked. UTR, untranslated region. (B) PCR analysis of genomic DNA from the WT and two Δ*pbg37* parasite clones. Predicted DNA fragment sizes are as follows: with primers P1 and P2, 1,902 bp from the WT only; with primers P1 and P3, 1,828 bp from the Δ*pbg37* line only; with primers P4 and P5, 1,552 bp from the Δ*pbg37* line only.

lines ($P < 0.0001$; Fig. 4A). In addition, deletion of *pbg37* had a substantial impact on the sex ratio, with the Δ*pbg37* lines having significantly higher mature female/male ratios (~6:1) than the WT parasite (~3:1) ($P < 0.0001$; Fig. 4B). Real-time RT-PCR analysis also demonstrated that the male-specific *pbs230p* mRNA level was significantly decreased in the two KO groups compared with the WT group ($P < 0.01$; Fig. S6), whereas the female-specific *pbs47* mRNA level was not significantly different among the three groups ($P > 0.05$). Whereas the gross morphologies of the male and female gametocytes in Giemsa-stained thin smears in the Δ*pbg37* lines were similar to those in WT parasites, deletion of *pbg37* influenced the development and maturation of microgametocytes. At 3 days p.i., the Δ*pbg37* parasites showed a significantly lower proportion of mature male gametocytes than the WT group ($P = 0.007$; Fig. 4C), whereas the proportions of female gametocytes in the Δ*pbg37* lines and the WT were not significantly different ($P > 0.05$; Fig. 4D). Furthermore, even though the mature microgametocytes appeared to be morphologically normal, they exhibited defects in exflagellation. When the same numbers of mature male gametocytes were compared between the Δ*pbg37* lines and the WT in an *in vitro* exflagellation assay, the Δ*pbg37* lines had an ~36% decrease in the number of exflagellation centers formed per field compared to that in the WT parasites ($P < 0.0001$; Fig. 4E). Altogether, these phenotypic analyses showed more severe developmental defects in male gametocytogenesis in the Δ*pbg37* lines.

We next determined the consequence of *pbg37* deletion on fertilization and subsequent development using an *in vitro* assay. Even when the same number of mature male gametocytes was used in the *in vitro* ookinete culture, the Δ*pbg37* lines showed an ~75% decrease in the ookinete number ($P < 0.0001$; Fig. 4F). There was an ~64% reduction in the total number of fertilized stages (zygotes, retorts, and ookinetes) in the two Δ*pbg37* lines, suggesting a defect in fertilization in the KO lines. At the end of ookinete culture, 84% of the zygotes in the WT parasites but only ~46% of those in the Δ*pbg37* lines developed into ookinetes, suggesting that zygote-to-ookinete development was also impaired in the Δ*pbg37* lines. Mosquitoes fed on mice infected with the

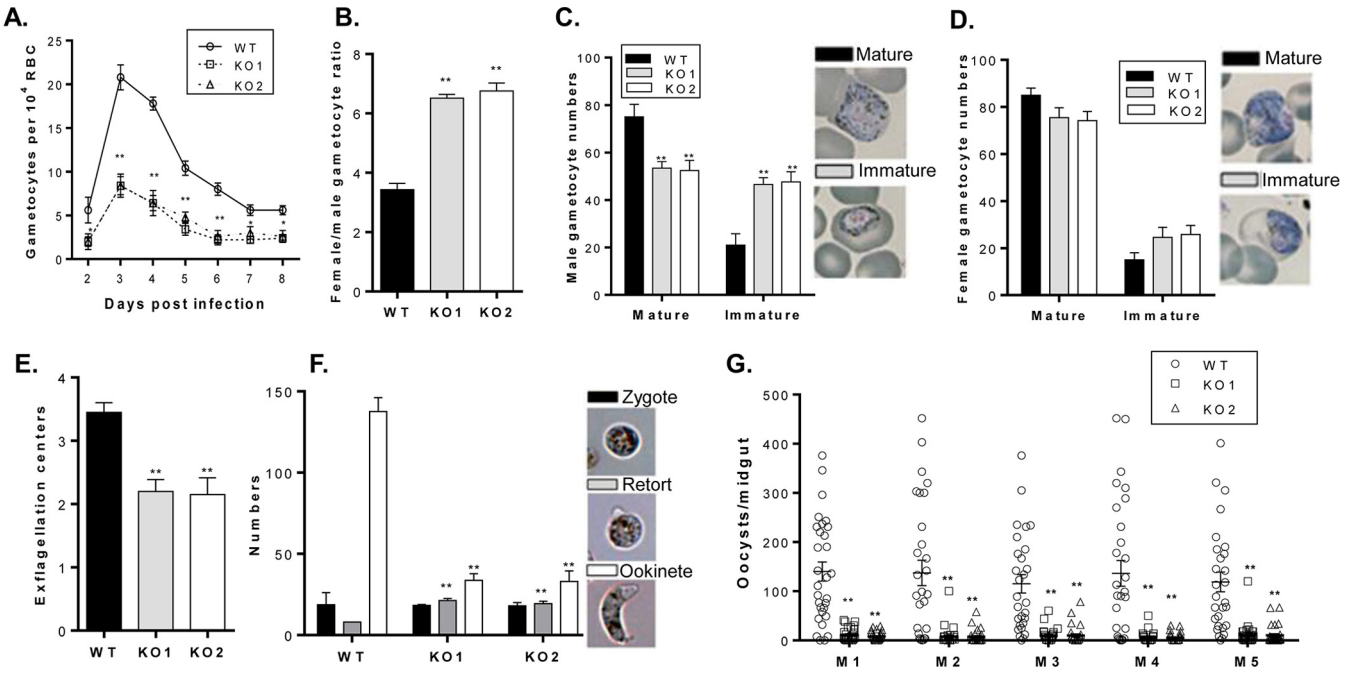

FIG 4 Phenotypic comparison of two Δ*pbg37* lines (KO1 and KO2) with the WT parasites. BALB/c mice were injected intraperitoneally with 1×10^6 WT or Δ*pbg37* parasites. (A) Daily gametocytemia (per 10^4 RBCs). (B) Female/male gametocyte ratios at day 3 p.i. (C) Numbers of mature and immature male gametocytes per 100 male gametocytes at day 3 p.i. (D) Numbers of mature and immature female gametocytes per 100 female gametocytes at day 3 p.i. (E) Numbers of exflagellation centers per microscopic field at a magnification of ×100 with equal numbers of mature male gametocytes. (F) Zygote, retort, and ookinete numbers in ookinete cultures from equal numbers of mature male gametocytes at day 3 p.i. The error bars in panels A to F indicate the mean ± SEM. (G) Midgut oocyst numbers per mosquito fed on either WT or Δ*pbg37* parasites. Horizontal bars indicate the mean values. **, statistical significance compared with the WT ($P < 0.01$). All data in the phenotypic analysis are from three independent experiments.

Δ*pbg37* lines showed a modest reduction in infection rate (~68.6% in the Δ*pbg37* groups) compared to the rate for those fed on mice infected with the WT parasites (92.5%) ($P < 0.0001$) (Table 1). Moreover, there was a ~92.1% reduction in the mean oocyst number per midgut in mosquitoes fed on mice infected with the Δ*pbg37* parasites (~10.7) compared to that in mosquitoes fed on mice infected with the WT (135.6) ($P < 0.0001$) (Fig. 4G; Table 1).

Anti-rPbg37 antibody has modest TB activity. The surface expression of Pbg37 in sexual stages prompted us to evaluate the TB potential of Pbg37 using both *in vitro* ookinete formation and direct mosquito feeding assays. Macrogametocytes and macrogametes were differentiated on the basis of surface staining of the latter with the Pbs21 antibody (Fig. S4B). Incubation of the WT *P. berghei* gametocytes with the anti-Pbg37 antiserum at 1:5, 1:10, and 1:50 dilutions led to 70% ($P < 0.0001$, *t* test), 65% ($P < 0.0001$, *t* test), and 60% ($P < 0.0001$, *t* test) decreases in the numbers of exflagellating males, respectively (Fig. 5A). Conversely, the anti-Pbg37 antibodies had no noticeable effect on the formation of macrogametes when examined at 15 min (Fig. S7A). At 2 h, however, when the majority (~99%) of the macrogametes were converted to zygotes in cultures with the control serum, ~65% of the macrogametes remained unfertilized in cultures with the anti-Pbg37 antiserum (Fig. S7B). As a result, the zygote numbers in cultures with anti-Pbg37 antiserum at 1:5, 1:10, and 1:50 dilutions were reduced by 69.6% ($P < 0.0001$, *t* test), 65.1% ($P < 0.0001$, *t* test), and 60.5% ($P < 0.0001$, *t* test), respectively, compared to the zygote numbers in cultures treated with the control serum (Fig. S7C). However, the anti-Pbg37 antiserum did not have a significant impact on the subsequent development of the parasite, as the zygote-to-ookinete conversion rates were not significantly different between cultures with the control and anti-Pbg37 sera at all dilutions tested (Fig. S7D). Only in the 1:5 dilution groups did we observe a 4.7% decrease in the ookinete conversion rate in cultures with the anti-Pbg37 antiserum compared to that in the control cultures ($P > 0.05$, *t* test). In total, the

TABLE 1 Prevalence of infection and oocyst numbers in mosquitoes fed on WT and Δ*pbg37* parasites

Mouse	No. of mosquito-infected mice/ total no. of dissected mice	Prevalence of infection (%)[a]	Mean prevalence (%)	Reduction in prevalence (%)[b]	Oocyst density[c]	SEM of oocyte density	Mean oocyst density	Reduction in oocyst density (%)[d]
WT			92.5				135.6	
1	28/30	93.3			150.1	19.3		
2	26/29	89.6			153.1	25.8		
3	27/28	96.4			119.5	18.9		
4	26/30	86.7			136.4	26.3		
5	28/27	96.4			119	20. 2		
KO1			69.5	23			11.5	91.5
1	22/30	73.3			15.3	2.4		
2	19/30	63.3			11.8	3.6		
3	20/28	71.4			13	2.6		
4	19/27	70.4			5.5	1.9		
5	20/29	69			11.9	4.3		
KO2			67.6	24.9			9.8	92.7
1	20/30	66.7			8.1	1.7		
2	19/29	65.5			7.4	2.5		
3	20/29	69			17.2	3.4		
4	19/29	65.5			5.8	1.6		
5	20/28	71.4			10.3	3.4		

[a]The prevalence of infection was calculated by the (number of mosquitoes with oocysts/total number of mosquitoes dissected in each group) × 100.
[b]The percent reduction of prevalence was calculated as the percent mean prevalence for the WT − the percent mean prevalence for the KO.
[c]Mean number of oocysts per mosquito midgut.
[d]The percent reduction in oocyst density was calculated as [(mean oocyst density for the WT − mean oocyst density for the KO)/mean oocyst density for the WT] × 100.

anti-Pbg37 antiserum at 1:5, 1:10, and 1:50 dilutions reduced ookinete formation in the *in vitro* assay by 71% ($P < 0.0001$), 65% ($P < 0.0001$), and 61% ($P < 0.0001$), respectively (Fig. 5B).

To test the TB potential of Pbg37 *in vivo*, mice were immunized with the rPbg37 fusion protein or the control Trx-His tag protein using our established immunization scheme, and then direct mosquito feeding with *Anopheles stephensi* mosquitoes was performed 14 days after the final immunization. Whereas rPbg37 immunization resulted in only a slight (9.23%) reduction in the prevalence of infected mosquitoes ($P =$

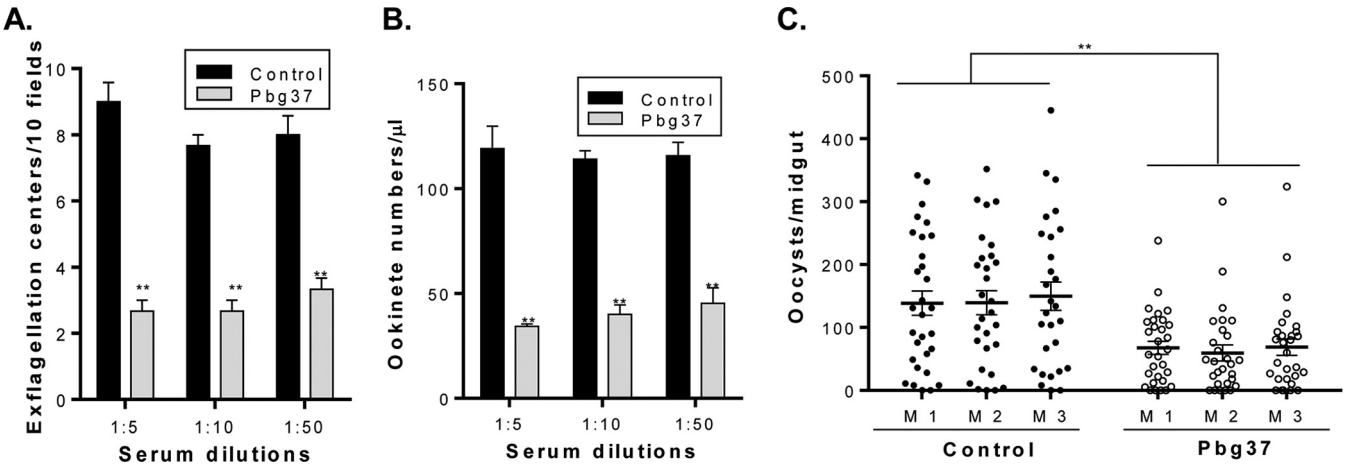

FIG 5 TB potential of rPbg37 with the *in vitro* ookinete formation assay and mosquito feeding assay. (A, B) WT *P. berghei*-infected blood collected at 3 days p.i. was incubated with the anti-rPbg37 serum or control serum, which was diluted with serum from naive mice at final dilutions of 1:5, 1:10, and 1:50. (A) Exflagellation centers in 10 microscopic fields. (B) Ookinete numbers after 24 h of *in vitro* culture. The data are representative of those from three independent experiments. (C) Midgut oocyst numbers in mosquitoes fed on rPbg37-immunized mice ($n = 3$) and control Trx-His-tagged protein-immunized mice ($n = 3$) at day 3 after infection with WT *P. berghei*. Data points represent midgut oocyst numbers in individual mosquitoes. Horizontal bars indicate the mean number of oocysts per midgut from mosquitoes fed on each mouse ($n = 30$). Error bars in all panels indicate the mean ± SEM. **, $P < 0.01$.

TABLE 2 Evaluation of the transmission-blocking effect of anti-rPbg37 serum in mosquito feeding assays[d]

Mouse	No. of mosquito-infected mice/total no. of dissected mice	Prevalence of infection (%)[a]	Mean prevalence (%)	Reduction in prevalence (%)[b]	Oocyst density[c]	SEM	Mean oocyst density
Control mice			93.1				153.2
M1	28/30	93.3			148.6	19.486	
M2	27/29	93.1			149.7	19.227	
M3	26/28	92.9			161.4	22.628	
rPbg37-immunized mice			83.9				78
M1	26/30	86.7			78.2	10.514	
M2	23/28	82.1			72.5	12.698	
M3	24/29	82.8		9.23	83.8	13.152	

[a]The prevalence of infection was calculated by the (number of mosquitoes with oocysts/total mosquitoes dissected in each group) × 100.
[b]The percent reduction of prevalence was calculated as the percent mean prevalence for the control − the percent mean prevalence for Pbg37-treated mice.
[c]Mean number of oocysts per mosquito midgut.
[d]The overall reduction in oocyst density was 49.1%, which was calculated as the [(mean oocyst density for the control − mean oocyst density for Pbg37-treated mice)/mean oocyst density for the control] × 100.

0.094) compared to that in those immunized with the control protein, it resulted in a 49.1% decrease of the oocyst density ($P < 0.0001$) (Fig. 5C; Table 2).

DISCUSSION

TBV has been regarded as an innovative and important tool for the elimination of malaria (6), especially that caused by *P. vivax*, which develops gametocytes to enable transmission earlier than the manifestation of clinical symptoms. However, the slim TBV development pipeline with few candidates for *P. vivax* demands greater efforts in TBV antigen discovery (30). Using *P. berghei* as a model, we identified a sexual-stage protein, Pbg37, and found that it was mainly expressed on the outer membranes of gametes, zygotes, and, to a lesser degree, retorts and ookinetes. Through functional studies we determined the importance of Pbg37 during gametocytogenesis and especially in the development of male gametocytes. We further showed that a small 63-amino-acid rPbg37 polypeptide expressed in bacteria was able to induce moderate TB activity in a mosquito feeding assay.

The availability of the proteomic data from the gametocyte stage to the ookinete stage provides unprecedented opportunities for TBV antigen discovery (31–34). *In silico* screening of TBV candidates using filters for potential surface localization and exclusive expression in sexual stages may yield very useful results worthy of future pursuance (35). For example, reverse genetics and bioinformatics allowed the identification of a number of putative secreted ookinete proteins (PSOPs), and several (e.g., PSOP7, -12, -25, -26) have been shown to be able to induce substantial TB immunity in *P. berghei* (28, 36–38). The current TBV candidates of parasite antigens include those from either the prefertilization stages (e.g., P230, P48/45, HAP2) or the postfertilization stages (e.g., P25, P28, chitinase). While the prefertilization antigens offer the advantage of boosting with immunization because of the existence of natural antibodies against these proteins in populations in which malaria is endemic, postfertilization expression of these antigens would allow an extended time of action for the blocking antibodies. Therefore, antigens expressed in both the pre- and postfertilization stages may induce stronger TB activities. In this sense, Pbg37 was unique, with surface localization in both prefertilization stages (gametes) and postfertilization stages (zygotes and ookinetes), albeit the expression level was gradually reduced during the transition from zygote to ookinete. In support of this, even a small N-terminal fragment of Pbg37 could induce substantial TB immunity, similar to the levels elicited by several PSOPs (28, 37). Detailed observation of the TB activity of the rPbg37 antiserum during *in vitro* ookinete culture studies demonstrated that the Pbg37 antiserum had a major inhibitory effect on the exflagellation process, since incubation of WT *P. berghei*-infected blood with rPbg37 antiserum substantially inhibited the formation of the exflagellation centers (>60%). Subsequent fertilization of the female gametes was also severely compromised, and the anti-Pbg37

antiserum blocked zygote formation by almost 70% compared with the control serum. Despite the presence of the Pbg37 protein associated with the plasma membranes of zygotes and retorts, the transition from zygote to ookinete was only slightly affected by the anti-Pbg37 antiserum.

The gametocyte stage is obligatory for the continued transmission of the malaria parasites in mosquitoes (39, 40). Sex allocation in *Plasmodium* is typically female biased (41, 42), but the underlying mechanism is not understood. Thus, understanding the biology of gametocytogenesis may lead to the identification of novel targets to interrupt parasite transmission. Through functional analysis, we showed that *pbg37* deletion resulted in a substantial reduction in gametocytemia, probably through inhibition of male gametocytogenesis. *pbg37* deletion affected not only the maturation process of the male gametocytes but also the function (exflagellation) of the mature male gametocytes, suggesting that Pbg37 is needed for the formation of the male gametes. Since *pbg37* deletion led to a sharp decrease in zygote formation, it is possible that Pbg37 is also involved in fertilization. Though female gametocytes did not appear to be affected, future validation is needed through hybridization experiments using a parasite line with defective female gametocytogenesis (e.g., *pbs47* deletion).

Despite the large numbers of proteins that have previously been screened for the ability to initiate a transmission-blocking response, only a few of the parasite-derived proteins are currently considered to be the lead candidates. Several current TBV antigens belong to the 6-Cys family, characterized by the presence of six positionally conserved cysteines in an ~120-residue domain (43). One of the challenges imposed by the presence of the cysteines in these proteins is the requirement to produce correctly folded native protein for the induction of TB immunity (44, 45). In this study, the use of a 63-amino-acid fragment, which was found to include enough epitopes to induce strong antibody responses with TB activity, is encouraging. Although the TB activity is much lower than that induced by P25, the Pbg37 peptide may still be a promising component for a TBV with multiple protein targets. With the high degree of conservation of this gene in *Plasmodium*, it is worthwhile to test the Pbg37 orthologs in human malaria parasites. Furthermore, efforts should be undertaken to identify a more suitable adjuvant for human trials (25) which could induce TB immunity as potent as the Freund's adjuvant used in a rodent malaria study.

MATERIALS AND METHODS

Mice, parasites, and mosquitoes. BALB/c mice aged 6 to 8 weeks were bought from the Beijing Animal Institute. The *P. berghei* ANKA strain was maintained by serial passages as described previously (28). Mouse infection was initiated by intraperitoneal injection of 1×10^7 *P. berghei*-infected red blood cells (iRBCs). *Anopheles stephensi* mosquitoes (Hor strain) were maintained in an insectary at 25°C and 50 to 80% humidity. All animal experiments were approved by the animal ethics committee of China Medical University.

Expression and purification of recombinant protein. For the expression of rPbg37, total RNA was extracted from purified gametocytes and RT-PCR was performed to amplify the *pbg37* fragment encoding amino acids 26 to 88 with primers *pbg37*-F and *pbg37*-R (see Table S1 in the supplemental material). The PCR product was digested with BamHI and NotI and cloned into pET32a(+). Expression of the rPbg37 in *Escherichia coli* Rosetta-gami B (DE3) cells was induced with 1 mM isopropyl-D-1-thiogalactoside (Sigma) at 20°C for 8 h, and rPbg37 fused with the thioredoxin (Trx) from the expression vector was purified on an Ni-NTA agarose column (Novagen). The Trx-His tag protein expressed from the empty vector pET32a(+) was similarly purified and used as a control for immunization. Purified recombinant proteins were dialyzed extensively in phosphate-buffered saline (PBS; pH 7.4) at 4°C overnight and analyzed on a 10% SDS-PAGE gel. While the rPbg37 fusion protein was used for immunization, the rPbg37 polypeptide was further purified from the Ni-NTA agarose column after digestion with enterokinase (Solarbio). The released rPbg37 polypeptide (~7 kDa) was concentrated, and its purity was determined by electrophoresis on a Tris-Tricine-SDS-PAGE gel.

Immunization of BALB/c mice. BALB/c mice ($n = 6$) were immunized with the rPbg37-Trx fusion protein (50 μg each) emulsified in complete Freund's adjuvant (Sigma) via subcutaneous injection. Two booster immunizations with the same rPbg37 fusion protein (25 μg/mouse) were done at a 2-week interval. The same immunization procedure was followed with another group of mice ($n = 6$) using the Trx control protein. Serum was collected and pooled for each group of mice on the day before each immunization and at 14 days after the final immunization.

ELISA was used to assess the antibody titers in the rPbg37 immunization group as previously described (28, 46). To estimate the endpoint titer of antiserum from the rPbg37-immunized group and the control group, 96-well plates were coated overnight at 4°C with the purified Pbg37 polypeptides at

5 μg/ml. Serum was serially diluted in 1% bovine serum albumin (BSA) in PBS from 1:2,000 to 1:128,000. The endpoint was defined as the highest dilution of the antiserum when the optical density (OD) value at 490 nm was above the average for the control antiserum plus 3 times the standard deviation.

Western blotting. To determine the expression of the Pbg37 protein in different development stages of the parasite, *P. berghei* schizonts, gametocytes, zygotes, and ookinetes were purified on Nycodenz gradients essentially as previously described (28). Proteins from purified parasites were extracted with 2% SDS containing protease inhibitors. To determine the localization of the Pbg37 protein in the cytosol or membrane fractions, purified gametocytes were separated into membrane and soluble protein fractions using a membrane protein isolation kit (Invent Biotechnologies). Briefly, purified gametocytes were treated with 0.15% saponin to lyse the erythrocytes, and parasites were collected by centrifugation and washed once with PBS. Pellets were lysed in buffer A with protease inhibitors (Roche) in a filter cartridge. After centrifugation at 14,000 rpm for 30 s, the pellets were resuspended and centrifuged at 3,000 rpm for 1 min. The supernatant was collected, centrifuged at 14,000 rpm for 10 min, and kept as the cytoplasmic protein fraction. The pellet (total membrane fraction) was resuspended in buffer B and centrifuged at 10,000 rpm for 20 min. The supernatant was then centrifuged again at 14,000 rpm for 30 min, and the pellet was collected as the membrane protein fraction. Protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific). Equal amounts of parasite proteins (10 μg) of the four parasite stages and equal amounts (30 μg) of the gametocyte cytosolic and membrane proteins were separated in a 10% SDS-PAGE gel under reducing conditions and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) at room temperature for 2 h and probed with mouse anti-rPbg37 antiserum and control serum against Trx (1:200). After three washes with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody. Protein bands were visualized by chemiluminescence using a Pierce ECL Western blotting kit.

IFA. The localization of Pbg37 in parasites was detected by IFA (47). Briefly, on day 4 postinfection, gametocytes were collected in PBS and kept on ice to avoid activation. To produce gametes, gametocyte-infected blood was taken from a mouse and immediately mixed with ookinete culture medium for 15 min at 25°C. Then, cultured parasites were washed twice with PBS. Both activated (gametes) and nonactivated gametocytes and other parasite stages (schizonts, zygote, retort, and ookinete) were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min at room temperature (47). Parasites were washed once with 0.15% glycine in PBS for 10 min. The fixed cells were either permeabilized with 0.1% Triton X-100 in PBS for 10 min and then blocked with 3% BSA–PBS for 60 min or blocked without permeabilization. Following another PBS wash, mouse anti-rPbg37 antiserum and control serum (diluted to 1:200 in 3% BSA–PBS) were added and the mixture was incubated at 37°C for 1 h. After washing the slides with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin antibody (Invitrogen) diluted to 1:500 in 3% BSA–PBS was added and the mixture was incubated for 1 h. To distinguish extracellular female gametes from intraerythrocytic female gametocytes, a monoclonal antibody against Pbs21 was used to show the surface staining of female gametes and ookinetes. Parasite nuclei were counterstained with 10 μl DAPI (4′,6-diamidino-2-phenylindole; Sigma). To observe the localization of Pbg37 under native conditions, unfixed parasites were collected in PBS or RPMI 1640 culture medium and IFA was performed as described above with anti-rPbg37 antiserum. IFA slides were mounted with ProLong Gold antifade reagent (Invitrogen) and observed under an Olympus BX53 microscope.

Generation of transgenic parasites. A double-crossover homologous recombination strategy was used to generate the Δ*pbg37* lines (48). The *pbg37* 5′ and 3′ flanking regions were amplified using primer pairs 5UTR-F–5UTR-R and 3UTR-F–3UTR-R, respectively (Table S1) and cloned into the vector to flank the human *dhfr* expression cassette. Ten micrograms of the plasmid was linearized by HindIII and XhoI digestion and electroporated into purified *P. berghei* schizonts using a Nucleofector system. After transfection, the parasites were mixed with 50 μl of complete culture medium and the mixture was inoculated into mice. Twenty-four hours later, parasites were selected with pyrimethamine (70 μg/ml) via the drinking water for mice. Two independent transfection experiments were performed to obtain different parasite clones. Parasite genomic DNA was extracted from infected blood to confirm *pbg37* gene deletion using integration-specific diagnostic PCR with primer pairs P1-P2, P1-P3, and P4-P5 (Table S1).

Phenotype analysis of the Δ*pbg37* parasites. To study the effect of the *pbg37* deletion on parasite development, we compared the phenotypes between two *pbg37* deletion clones (KO1 and KO2) and the WT parasites. For each group, five mice were used; each was injected intraperitoneally with 1×10^6 iRBCs of the respective parasite clones. Parasitemia and gametocytemia were monitored daily by microscopic examination of Giemsa-stained thin blood films. In each mouse, a total of 100 mature gametocytes were differentiated into male and female gametocytes on the basis of the morphological characteristics to determine the gametocyte sex ratio. Female gametocytes are characterized by a relatively small nucleus with a nucleolus and concentrated pigment. In male gametocytes, the nucleus is larger without a distinctive nucleolus, and the pigment is more diffuse. In Giemsa-stained blood films, male gametocytes are pink cells, whereas female gametocytes are more violet or bluish (17). The proportion of mature gametocytes in the total gametocyte population was also estimated with at least 100 gametocytes. The gametocyte density and the proportion of mature male gametocytes were used to calculate the volume of infected blood to be used for exflagellation and ookinete formation experiments. Exflagellation of male gametocytes and formation of ookinetes were monitored using *in vitro* cultures (48, 49). Briefly, infected blood containing an equal number of mature male gametocytes was mixed with the ookinete culture medium and

incubated at 25°C for 15 min to induce gamete formation. The number of exflagellation centers in each ×100 magnification field was counted under a phase-contrast microscope. A total of 20 fields were counted per mouse. To observe ookinete formation, blood samples were incubated in ookinete culture medium (RPMI 1640, 50 mg/liter penicillin, 50 mg/liter streptomycin, 100 mg/liter neomycin, 25% [vol/vol] heat-inactivated fetal calf serum, 6 U/ml heparin, pH 8.3) at 19°C for 24 h, and ookinetes were monitored by IFA using the monoclonal antibody against Pbs21.

To determine whether *pbg37* KO affected the sex ratio of the gametocytes, total RNA was extracted from 1×10^6 gametocytes of the WT, KO1, and KO2 parasites with the TRIzol reagent (Invitrogen). cDNA was synthesized from 1 μg of each RNA sample using a TaKaRa RNA PCR kit. The expression levels of the male-specific *pbs230p* and the female-specific *pbs47* genes were quantified by real-time RT-PCR, with the expression of the *hsp70* gene used as the reference. Real-time RT-PCR mixtures consisted of 2 μl cDNA, 5 μl SYBR green fast master mix, 0.5 μl each of the forward and reverse primers (Table S1), and 2 μl diethyl pyrocarbonate-treated water. Analysis was performed using an Applied Biosystems 7500 machine with the following cycling conditions: denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Three replicates were used for each mouse. The relative quantitation of gene expression was done following an established protocol (50).

The infectivity of the parasites to mosquitoes was evaluated in a direct mosquito feeding experiment. On day 3 p.i., when asexual parasitemia had reached 5 to 7%, 4-day-old starved *A. stephensi* mosquitoes (50 per mouse) were allowed to feed on the infected mice for 1 h. Fully engorged mosquitoes were kept for 12 days, and approximately 30 mosquitoes of each group were dissected. Midguts were stained with 0.5% mercurochrome for 10 min to determine the oocyst number per positive midgut and the mosquito infection rate (the number of infected mosquitoes/the number of total mosquitoes) (51).

Quantification of TB activity. The TB potential of Pbg37 was assessed using an *in vitro* ookinete formation assay and a direct mosquito feeding assay. For the ookinete formation assay, mice were infected with an intraperitoneal injection of 1×10^6 WT *P. berghei* iRBCs after treatment with phenylhydrazine. At 3 days p.i., anti-rPbg37 serum or the control serum was diluted at 1:5, 1:10, and 1:50 with the ookinete culture medium and mixed with 10 μl of infected mouse blood in a total volume of 50 μl. The exflagellation of male gametocytes was quantified as described above (28). Samples were examined at 15 min, 2 h, 12 h, and 24 h to count the numbers of macrogametes, zygotes, retorts, and ookinetes on the basis of their morphological features after IFA with the monoclonal antibody against Pbs21 and DAPI staining of the nucleus.

For mosquito feeding experiments, mice were immunized with rPbg37 or the control Trx-His tag protein as described above. On day 14 after the final immunization, mice were infected with 1×10^6 parasites. At 3 days after infection, starved female *A. stephensi* mosquitoes were allowed to feed on the mice (~50 mosquitoes/mouse). The oocyst density and mosquito infection rate were determined as described above (28).

Statistical analyses. Statistical analyses were carried out using SPSS software, version 17.0 (SPSS Inc., USA). Student's *t* test was used for statistical comparison between groups of asexual parasitemia, gametocytes, sex ratio, exflagellation, and ookinete numbers. The Mann-Whitney U test was employed to analyze oocyst density (oocyst number per midgut), while Fisher's exact test was used to analyze infection prevalence. All data are presented as the mean ± standard error of the mean (SEM). A *P* value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00785-17.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grants (R01AI099611 to Y.C. and R01AI104946 to L.C.), by the National Science Foundation of China (81501769 to M.W. and 81429004 to L.C.), and the Liaoning Natural Science Foundation (2015020747).

Pbs21-specific monoclonal antibody clone 13.1 was a gift from Hiroyuki Matsuoka.

We declare that we have no competing interests.

REFERENCES

1. WHO. 2017. World malaria report 2017. WHO, Geneva, Switzerland.
2. Feachem RG, Phillips AA, Targett GA, Snow RW. 2010. Call to action: priorities for malaria elimination. Lancet 376:1517–1521. https://doi.org/10.1016/S0140-6736(10)61500-0.
3. Duru V, Witkowski B, Menard D. 2016. Plasmodium falciparum resistance to artemisinin derivatives and piperaquine: a major challenge for malaria elimination in Cambodia. Am J Trop Med Hyg 95:1228–1238. https://doi.org/10.4269/ajtmh.16-0234.
4. Ranson H, Lissenden N. 2016. Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. Trends Parasitol 32:187–196. https://doi.org/10.1016/j.pt.2015.11.010.
5. Olotu A, Fegan G, Wambua J, Nyangweso G, Leach A, Lievens M, Kaslow DC, Njuguna P, Marsh K, Bejon P. 2016. Seven-year efficacy of RTS,S/AS01 malaria vaccine among young African children. N Engl J Med 374:2519–2529. https://doi.org/10.1056/NEJMoa1515257.
6. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, Doumbo OK, Greenwood B, Hall BF, Levine MM, Mendis K, Newman RD,

Plowe CV, Rodriguez MH, Sinden R, Slutsker L, Tanner M. 2011. A research agenda to underpin malaria eradication. PLoS Med 8:e1000406. https://doi.org/10.1371/journal.pmed.1000406.
7. Carter R. 2001. Transmission blocking malaria vaccines. Vaccine 19:2309–2314. https://doi.org/10.1016/S0264-410X(00)00521-1.
8. Tsuboi T, Tachibana M, Kaneko O, Torii M. 2003. Transmission-blocking vaccine of vivax malaria. Parasitol Int 52:1–11. https://doi.org/10.1016/S1383-5769(02)00037-5.
9. Lavazec C, Bourgouin C. 2008. Mosquito-based transmission blocking vaccines for interrupting Plasmodium development. Microbes Infect 10:845–849. https://doi.org/10.1016/j.micinf.2008.05.004.
10. Arevalo-Herrera M, Solarte Y, Zamora F, Mendez F, Yasnot MF, Rocha L, Long C, Miller LH, Herrera S. 2005. Plasmodium vivax: transmission-blocking immunity in a malaria-endemic area of Colombia. Am J Trop Med Hyg 73(5 Suppl):38–43.
11. Kapulu MC, Da DF, Miura K, Li Y, Blagborough AM, Churcher TS, Nikolaeva D, Williams AR, Goodman AL, Sangare I, Turner AV, Cottingham MG, Nicosia A, Straschil U, Tsuboi T, Gilbert SC, Long CA, Sinden RE, Draper SJ, Hill AV, Cohuet A, Biswas S. 2015. Comparative assessment of transmission-blocking vaccine candidates against Plasmodium falciparum. Sci Rep 5:11193. https://doi.org/10.1038/srep11193.
12. Wu Y, Sinden RE, Churcher TS, Tsuboi T, Yusibov V. 2015. Development of malaria transmission-blocking vaccines: from concept to product. Adv Parasitol 89:109–152. https://doi.org/10.1016/bs.apar.2015.04.001.
13. Barr PJ, Green KM, Gibson HL, Bathurst IC, Quakyi IA, Kaslow DC. 1991. Recombinant Pfs25 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in experimental animals. J Exp Med 174:1203–1208. https://doi.org/10.1084/jem.174.5.1203.
14. Saxena AK, Wu Y, Garboczi DN. 2007. Plasmodium p25 and p28 surface proteins: potential transmission-blocking vaccines. Eukaryot Cell 6:1260–1265. https://doi.org/10.1128/EC.00060-07.
15. Miura K, Keister DB, Muratova OV, Sattabongkot J, Long CA, Saul A. 2007. Transmission-blocking activity induced by malaria vaccine candidates Pfs25/Pvs25 is a direct and predictable function of antibody titer. Malar J 6:107. https://doi.org/10.1186/1475-2875-6-107.
16. Eksi S, Czesny B, van Gemert GJ, Sauerwein RW, Eling W, Williamson KC. 2006. Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. Mol Microbiol 61:991–998. https://doi.org/10.1111/j.1365-2958.2006.05284.x.
17. van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, Dodemont HJ, Stunnenberg HG, van Gemert GJ, Sauerwein RW, Eling W. 2001. A central role for P48/45 in malaria parasite male gamete fertility. Cell 104:153–164. https://doi.org/10.1016/S0092-8674(01)00199-4.
18. Tachibana M, Wu Y, Iriko H, Muratova O, MacDonald NJ, Sattabongkot J, Takeo S, Otsuki H, Torii M, Tsuboi T. 2011. N-terminal prodomain of Pfs230 synthesized using a cell-free system is sufficient to induce complement-dependent malaria transmission-blocking activity. Clin Vaccine Immunol 18:1343–1350. https://doi.org/10.1128/CVI.05104-11.
19. Mori T, Kuroiwa H, Higashiyama T, Kuroiwa T. 2006. Generative cell specific 1 is essential for angiosperm fertilization. Nat Cell Biol 8:64–71. https://doi.org/10.1038/ncb1345.
20. Wong JL, Leydon AR, Johnson MA. 2010. HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. PLoS Genet 6:e1000882. https://doi.org/10.1371/journal.pgen.1000882.
21. Blagborough AM, Sinden RE. 2009. Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. Vaccine 27:5187–5194. https://doi.org/10.1016/j.vaccine.2009.06.069.
22. Lavazec C, Boudin C, Lacroix R, Bonnet S, Diop A, Thiberge S, Boisson B, Tahar R, Bourgouin C. 2007. Carboxypeptidases B of Anopheles gambiae as targets for a Plasmodium falciparum transmission-blocking vaccine. Infect Immun 75:1635–1642. https://doi.org/10.1128/IAI.00864-06.
23. Raz A, Dinparast Djadid N, Zakeri S. 2013. Molecular characterization of the carboxypeptidase B1 of Anopheles stephensi and its evaluation as a target for transmission-blocking vaccines. Infect Immun 81:2206–2216. https://doi.org/10.1128/IAI.01331-12.
24. Dinglasan RR, Kalume DE, Kanzok SM, Ghosh AK, Muratova O, Pandey A, Jacobs-Lorena M. 2007. Disruption of Plasmodium falciparum development by antibodies against a conserved mosquito midgut antigen. Proc Natl Acad Sci U S A 104:13461–13466. https://doi.org/10.1073/pnas.0702239104.
25. Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, Fay MP, Narum D, Rausch K, Miles AP, Aebig J, Orcutt A, Muratova O, Song G, Lambert L, Zhu D, Miura K, Long C, Saul A, Miller LH, Durbin AP. 2008. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS One 3:e2636. https://doi.org/10.1371/journal.pone.0002636.
26. Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, Miura K, Long CA, Lambert L, Miles AP, Wang J, Stowers A, Miller LH, Saul A. 2005. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine 23:3131–3138. https://doi.org/10.1016/j.vaccine.2004.12.019.
27. Talaat KR, Ellis RD, Hurd J, Hentrich A, Gabriel E, Hynes NA, Rausch KM, Zhu D, Muratova O, Herrera R, Anderson C, Jones D, Aebig J, Brockley S, MacDonald NJ, Wang X, Fay MP, Healy SA, Durbin AP, Narum DL, Wu Y, Duffy PE. 2016. Safety and immunogenicity of Pfs25-EPA/Alhydrogel(R), a transmission blocking vaccine against Plasmodium falciparum: an open label study in malaria naive adults. PLoS One 11:e0163144. https://doi.org/10.1371/journal.pone.0163144.
28. Zheng W, Kou X, Du Y, Liu F, Yu C, Tsuboi T, Fan Q, Luo E, Cao Y, Cui L. 2016. Identification of three ookinete-specific genes and evaluation of their transmission-blocking potentials in Plasmodium berghei. Vaccine 34:2570–2578. https://doi.org/10.1016/j.vaccine.2016.04.011.
29. Bousema T, Drakeley C. 2011. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clin Microbiol Rev 24:377–410. https://doi.org/10.1128/CMR.00051-10.
30. Mueller I, Shakri AR, Chitnis CE. 2015. Development of vaccines for Plasmodium vivax malaria. Vaccine 33:7489–7495. https://doi.org/10.1016/j.vaccine.2015.09.060.
31. Hall N, Carlton J. 2005. Comparative genomics of malaria parasites. Curr Opin Genet Dev 15:609–613. https://doi.org/10.1016/j.gde.2005.09.001.
32. Wass MN, Stanway R, Blagborough AM, Lal K, Prieto JH, Raine D, Sternberg MJ, Talman AM, Tomley F, Yates J, III, Sinden RE. 2012. Proteomic analysis of Plasmodium in the mosquito: progress and pitfalls. Parasitology 139:1131–1145. https://doi.org/10.1017/S0031182012000133.
33. Miao J, Chen Z, Wang Z, Shrestha S, Li X, Li R, Cui L. 2017. Sex-specific biology of the human malaria parasite revealed from the proteomes of mature male and female gametocytes. Mol Cell Proteomics 16:537–551. https://doi.org/10.1074/mcp.M116.061804.
34. Lasonder E, Rijpma SR, van Schaijk BC, Hoeijmakers WA, Kensche PR, Gresnigt MS, Italiaander A, Vos MW, Woestenenk R, Bousema T, Mair GR, Khan SM, Janse CJ, Bartfai R, Sauerwein RW. 2016. Integrated transcriptomic and proteomic analyses of P. falciparum gametocytes: molecular insight into sex-specific processes and translational repression. Nucleic Acids Res 44:6087–6101. https://doi.org/10.1093/nar/gkw536.
35. Chaurio RA, Pacheco MA, Cornejo OE, Durrego E, Stanley CE, Jr, Castillo AI, Herrera S, Escalante AA. 2016. Evolution of the transmission-blocking vaccine candidates Pvs28 and Pvs25 in Plasmodium vivax: geographic differentiation and evidence of positive selection. PLoS Negl Trop Dis 10:e0004786. https://doi.org/10.1371/journal.pntd.0004786.
36. Ecker A, Bushell ES, Tewari R, Sinden RE. 2008. Reverse genetics screen identifies six proteins important for malaria development in the mosquito. Mol Microbiol 70:209–220. https://doi.org/10.1111/j.1365-2958.2008.06407.x.
37. Sala KA, Nishiura H, Upton LM, Zakutansky SE, Delves MJ, Iyori M, Mizutani M, Sinden RE, Yoshida S, Blagborough AM. 2015. The Plasmodium berghei sexual stage antigen PSOP12 induces anti-malarial transmission blocking immunity both in vivo and in vitro. Vaccine 33:437–445. https://doi.org/10.1016/j.vaccine.2014.11.038.
38. Zheng W, Liu F, He Y, Liu Q, Humphreys GB, Tsuboi T, Fan Q, Luo E, Cao Y, Cui L. 2017. Functional characterization of Plasmodium berghei PSOP25 during ookinete development and as a malaria transmission-blocking vaccine candidate. Parasit Vectors 10:8. https://doi.org/10.1186/s13071-016-1932-4.
39. Williamson KC, Keister DB, Muratova O, Kaslow DC. 1995. Recombinant Pfs230, a Plasmodium falciparum gametocyte protein, induces antisera that reduce the infectivity of Plasmodium falciparum to mosquitoes. Mol Biochem Parasitol 75:33–42. https://doi.org/10.1016/0166-6851(95)02507-3.
40. Janse CJ, Franke-Fayard B, Waters AP. 2006. Selection by flow-sorting of genetically transformed, GFP-expressing blood stages of the rodent malaria parasite, Plasmodium berghei. Nat Protoc 1:614–623. https://doi.org/10.1038/nprot.2006.88.
41. Robert V, Read AF, Essong J, Tchuinkam T, Mulder B, Verhave JP, Carnevale P. 1996. Effect of gametocyte sex ratio on infectivity of Plasmodium falciparum to Anopheles gambiae. Trans R Soc Trop Med Hyg 90:621–624. https://doi.org/10.1016/S0035-9203(96)90408-3.

42. Paul RE, Brey PT, Robert V. 2002. Plasmodium sex determination and transmission to mosquitoes. Trends Parasitol 18:32–38. https://doi.org/10.1016/S1471-4922(01)02122-5.
43. van Dijk MR, van Schaijk BC, Khan SM, van Dooren MW, Ramesar J, Kaczanowski S, van Gemert GJ, Kroeze H, Stunnenberg HG, Eling WM, Sauerwein RW, Waters AP, Janse CJ. 2010. Three members of the 6-Cys protein family of Plasmodium play a role in gamete fertility. PLoS Pathog 6:e1000853. https://doi.org/10.1371/journal.ppat.1000853.
44. Kocken CH, Jansen J, Kaan AM, Beckers PJ, Ponnudurai T, Kaslow DC, Konings RN, Schoenmakers JG. 1993. Cloning and expression of the gene coding for the transmission blocking target antigen Pfs48/45 of Plasmodium falciparum. Mol Biochem Parasitol 61:59–68. https://doi.org/10.1016/0166-6851(93)90158-T.
45. Outchkourov NS, Roeffen W, Kaan A, Jansen J, Luty A, Schuiffel D, van Gemert GJ, van de Vegte-Bolmer M, Sauerwein RW, Stunnenberg HG. 2008. Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice. Proc Natl Acad Sci U S A 105:4301–4305. https://doi.org/10.1073/pnas.0800459105.
46. Miura K, Orcutt AC, Muratova OV, Miller LH, Saul A, Long CA. 2008. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. Vaccine 26:193–200. https://doi.org/10.1016/j.vaccine.2007.10.064.
47. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF, McFadden GI. 2004. Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 137:13–21. https://doi.org/10.1016/j.molbiopara.2004.05.009.
48. Tewari R, Straschil U, Bateman A, Bohme U, Cherevach I, Gong P, Pain A, Billker O. 2010. The systematic functional analysis of Plasmodium protein kinases identifies essential regulators of mosquito transmission. Cell Host Microbe 8:377–387. https://doi.org/10.1016/j.chom.2010.09.006.
49. Guttery DS, Poulin B, Ferguson DJ, Szoor B, Wickstead B, Carroll PL, Ramakrishnan C, Brady D, Patzewitz EM, Straschil U, Solyakov L, Green JL, Sinden RE, Tobin AB, Holder AA, Tewari R. 2012. A unique protein phosphatase with kelch-like domains (PPKL) in Plasmodium modulates ookinete differentiation, motility and invasion. PLoS Pathog 8:e1002948. https://doi.org/10.1371/journal.ppat.1002948.
50. Shrestha S, Li X, Ning G, Miao J, Cui L. 2016. The RNA-binding protein Puf1 functions in the maintenance of gametocytes in Plasmodium falciparum. J Cell Sci 129:3144–3152. https://doi.org/10.1242/jcs.186908.
51. Beetsma AL, van de Wiel TJ, Sauerwein RW, Eling WM. 1998. Plasmodium berghei ANKA: purification of large numbers of infectious gametocytes. Exp Parasitol 88:69–72. https://doi.org/10.1006/expr.1998.4203.